A REQUIREMENT FOR TRYPSIN-SENSITIVE CELL-SURFACE COMPONENTS FOR CELL-CELL INTERACTIONS OF EMBRYONIC NEURAL RETINA CELLS

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ABSTRACT

A quantiative assay was used to measure the rate of collection of a population of embryonic neural retina cells to the surface of cell aggregates. The rate of collection of freshly trypsinized cells was limited in the initial stages by the rate of replacement of trypsin-sensitive cell-surface components. When cells were preincubated, or "recovered," and then added to cell aggregates, collection occurred at a linear rate and was independent of protein and glycoprotein synthesis. The adhesion of recovered cells was temperature and energy dependent, and was reversibly inhibited by cytochalasin B. Colchicine had little effect on collection of recovered cells.

Antiserum directed against recovered cell membranes was shown to bind to recovered cells by indirect immunofluorescence. The antiserum also was shown to inhibit collection of recovered cells to aggregates, suggesting that at least some of the antigens identified might be involved in the adhesion process. The inhibitory effect of the antiserum was dose dependent. Freshly trypsinized cells absorbed neither the immunofluorescence activity nor the adhesion-inhibiting activity. Recovered cells absorbed away both activities.

In specificity studies, dorsal neural retina cells adhered to aggregates of ventral optic tectum in preference to aggregates of dorsal optic tectum. The adhesive specificity of the dorsal retina cells was less sensitive to trypsin than the adhesive specificity of ventral retina cells which adhered preferentially to dorsal tectal aggregates only after a period of recovery.

KEY WORDS embryonic cells · cell-surface recognition · trypsin-sensitive components

Many cellular processes require specific cell-surface receptors. Cell-surface recognition molecules are important for the fertilization reaction and as hormone receptors, as recognition sites in immune response phenomena, and, most likely, as participants in cell-cell interactions. Although direct knowledge of cell adhesion-related molecules is missing, ample experimental evidence exists suggesting a precise species- and tissue- specificity of embryonic cell interactions (34, 11, 19, 20, 25, 29). Correlated with this specificity, in some cases, is an antigenic specificity that parallels the adhesion behavior (9, 19). The relationship between

specific antigens and adhesive specificity is still circumstantial but does offer an approach for investigating the ability of tissues to organize themselves during development.

Intuitively, it is difficult to imagine morphogenesis in the absence of some kind of cell-surface recognition phenomenon. The problem has been to find experimental approaches that can answer questions concerning cell-cell interactions. A number of laboratories are using the phenomenon of cell aggregation as a paradigm to study the formation of cell adhesions. This in vitro process, though simplified when compared to morphogenetic processes in vivo, is in itself complex and appears to involve a series of reactions that are interrelated (17, 27, 31, 40). Some of these reactions can be separated for analytical purposes. For example, one could study binding properties between two cell surfaces, cell shape changes, membrane translational diffusional properties, metabolic properties, cell sorting out phenomena, cell junctions, or macromolecular factors. These are but a few of the possible contributors to the phenomenon known as cell adhesion or cell-cell interaction. Perhaps the most rewarding experimental approaches are attempts to focus on a single aspect of the complicated process.

Here we present data which describe the recovery or repair of embryonic neural retina cell-surfaces after trypsinization. After trypsin dissociation, several hours are necessary for cell-surface recovery before adhesive interactions occur at a high rate. During this time, materials required for adhesion appear at the cell-surface. Repair is confirmed by indirect immunofluorescence in that antiserum directed against "recovered" cell membranes binds to the surface of recovered cells. Its activity cannot be removed by freshly trypsinized cells, but the activity is removed by absorption with recovered cells. Aggregation experiments suggest that some of these antigens participate in the adhesion process: in kinetic assays, specifically absorbed antiserum binds to recovered cells and then inhibits the collection of these cells to the surface of collecting aggregates. The inhibition is specific and shows dose response properties which suggest a quantitative requirement for adhesive sites in the collection process.

The specificity of cell-cell recognition events is also known to be affected by trypsin. Cells of the dorsal neural retina adhere preferentially to aggregates of ventral optic tectum, and cells of the ventral neural retina adhere preferentially to aggregates of the dorsal optic tectum. Although both specificities are sensitive to trypsin, the ventral neural retina has a much greater sensitivity than the dorsal neural retina.

These data support the concept that specific cell-surface sites are required for cell adhesion. These sites are destroyed by trypsin and must be replaced, before the capacity for adhesion returns. These data also support an immunological approach for identification of cell-surface molecules that participate in adhesion.

MATERIALS AND METHODS

Cell Culture Conditions

Embryonic chick neural retinas (10 days) were dissected and dissociated with crystallized trypsin (Tryptar, Armour Pharmaceutical Co., Chicago, Ill.) after the methods of Moscona (24). Unless otherwise indicated, the tissues were incubated in trypsin at a strength of 4,000 U (BAEE)/ml (0.03%) in calcium-magnesium-free Tyrode's solution. After a 20-min incubation at 37°C, the tissues were washed three times in Eagle's minimal essential medium (MEM) plus 50 U/ml penicillin and 50 μg/ml streptomycin (EI medium) and dispersed into single-cell suspensions in EI plus DNase (20 μg/ml, Worthington Biochemical Corp., Freehold, N. J.). The cell suspensions were washed twice in EI-DNase, after which more than 95% of the cells excluded trypan blue. To recover cells before use in aggregation studies, cells ($0.5–1 \times 10^6$ cells/ml) were placed into 250-ml Erlenmeyer flasks in EI medium that had been preconditioned (from 24 h of culture over monolayers) or that contained 2% fetal calf serum. The suspensions were rotated at 115 rpm for 4–5 h; the cells were then harvested and placed into EI-DNase. The resuspended cells, now referred to as recovered cells, were viable at the 95% level as judged by trypan blue exclusion.

Adhesion Assay

The rate of collection of cells to the surface of aggregates was measured by a method previously described (18). Briefly, this assay measures the rate of collection of tritium-labeled single cells to the surface of cell aggregates in suspension culture. Conditions are adjusted so that hundreds of aggregates are available for collection; single cells are few in number, so there is very little opportunity for them to adhere to one another. The assay is designed so that replicates can be stopped at any time for a determination of the percentage of cells in suspension that have adhered to the surface of the collecting aggregates per unit time. Aggregates containing collected cells are separated from single cells by means of a custom-made filtering apparatus (18). Using a double-

label technique with the aggregates containing incorporated [^{14}C]leucine for internal standardization, we routinely obtain results showing a SE of ±4% in terms of the percentage of cells adhering to collecting aggregates in three replicate flasks per unit time. For each experiment shown below in Results, the mean and standard error are given. It should be noted that the collection assay measures interactions that result in adhesions stable enough to resist processing procedures. This working definition of adhesion does not necessarily examine all adhesion events (17).

Membrane Isolation

Cell membranes were isolated by swelling cells in hypotonic saline (0.01 M Tris, 0.01 M NaCl, 0.0015 M $MgCl_2$, pH 7.4 with 25 μg/ml DNase) for 10 min at 4°C; the cells were then homogenized by the Dounce method (Kontes Co., Vineland, N. J.) (25 strokes with the A, and 25 with the B pestle), and nuclei were centrifuged over a 0.25 M sucrose pad at 800 rpm in an International PR2 refrigerated centrifuge (International Equipment Co., Boston, Mass.) at 4°C. The particulate material in the sueprnate was precipitated in a Beckman J21B centrifuge (Spinco Div., Beckman Instruments, Palo Alto, Calif.) with a J20 rotor at 20,000 rpm for 30 min. The precipitate was resuspended in phosphate buffer (0.2 M PO_4, pH 7.2) and sonicated. This fraction contained about a fivefold purification of plasma membranes on the basis of enzyme marker assays (23).

Immunological Procedures

Rabbits received injections of crude cell membranes (1–2 mg protein) obtained by the membrane isolation procedure given above. The membrane fraction was injected in multiple intradermal sites in Freund's complete adjuvant; after 4 wk, the rabbits were boosted intravenously with freshly prepared membrane (0.2–0.5 mg protein) sonicated in Dulbecco's phosphate-buffered saline (PBS). The antiserum was absorbed repeatedly with freshly trypsinized cells (1:1 cell:serum volume) until it no longer reacted with freshly trypsinized cells. Fluorescein isothiocyanate (FITC) conjugated goat antirabbit Ig (Meloy Laboratories Inc., Springfield, Va.) was also preabsorbed with chick neural retina cells for 20 min on ice. The procedures of Goldschneider and Moscona (9) were followed for the indirect immunofluorescence microtiter assay. Sera from two rabbits were studied; except for titer differences, the biological properties were similar. In making Fab fragments (5) the two sera were combined.

Labeling of Cells

For aggregate collection experiments, cells to be collected were labeled with [^{3}H]leucine (10 μCi/ml, sp act 40–50 Ci/mmol) in leucine-free Eagle's MEM overnight before tissue dissociation. Cells for collecting aggregates were labeled in [^{14}C]leucine (0.05 μCi/ml, sp act 50 mCi/mmol) for 1–2 h before dissociation of tissues. Labeled amino acids were purchased from Schwarz/Mann Div. (Becton, Dickinson & Co., Orangeburg, N. Y.) or from New England Nuclear (Boston, Mass.).

RESULTS

Recovery of Cell Surfaces as Seen by Aggregation Experiments

The collection of cells to the surface of aggregates was measured under rotary culture conditions (18, 24, 29). In processing the collecting aggregates, loosely attached or perhaps "reversibly attached" cells (17, 40) were removed by washing procedures. The intent of the assay was to retain on the surface of aggregates only those cells that remained firmly attached. We first asked whether this assay system could distinguish between freshly trypsinized cells and recovered or repaired cells in their ability to be collected to aggregate surfaces. Recovered cells were cells that had been trypsin-dissociated and then incubated in dilute suspension (about 0.5–1 × 10^6 cells/ml) at 115 rpm for 4–5 h. We observed (Fig. 1) that

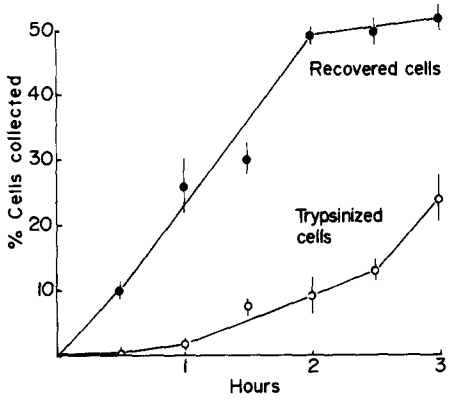

FIGURE 1 Adhesion of freshly trypsinized cells vs. recovered cells to the surface of collecting aggregates. All cells are from 10-day embryonic chick neural retina tissues. The rate of collection of recovered cells is linear for the first 2 h (linear regression analyses on more than 20 experiments similar to that shown indicate that the rate is linear with confidence limits at the 95–99% level). The rate of collection of trypsinized cells is nonlinear for the first 3–4 h. The initial rate is low and depends to some extent on the concentration of trypsin used for tissue dissociation. Recovered cells (1.2 × 10^5 cells/flask; 0.2 dpm/cell); freshly trypsinized cells (1.4 × 10^5 cells/flask; 0.25 dpm/cell). Each time-point represents the mean and SE of three replicate flasks containing about 500 collecting aggregates. Data are calculated as previously described (18).

recovered cells adhered to collecting aggregates at a linear rate whereas the collection rate for trypsinized cells was initially much lower and was nonlinear. This indicated to us that materials important in the adhesion process were replaced during cell-surface repair. Although we had no way of knowing whether the apparent recovery observed was complete, we now had a way to examine at least some aspects of the repair process.

Recovery vs. Adhesion as Processes Affected by Inhibitors

The ability to distinguish between processes involved in repair and processes involved in adhesion could now be studied. A number of inhibitors were tested for their effect on recovery (Table I) and for their effect on recovered cells (Table II). In the recovery tests, inhibitors were included in the recovery medium. As shown in Table I, cycloheximide inhibited collection of treated cells by about 50% when compared to an untreated control. The concentration of cycloheximide used (5 μg/ml) inhibited amino acid incorporation by 92 ± 2% during a 1-h treatment. If cells were given a chance to recover before cycloheximide treatment (Table II), the cycloheximide had little or no effect on the rate of collection for 2 h. We have previously shown that long treatments with cycloheximide eventually inhibit collection of recovered cells (18).

A similar pattern of inhibition was found with DON (6-diazo-5-oxo-L-norleucine), a glutamine analog that is thought to inhibit mucopolysaccharide synthesis (37, 39). Recovery was partially affected by DON (Table I), and collection of recovered cells was unaffected by DON at a concentration that inhibited incorporation of [^{35}S]sulfate into mucopolysaccharides by about 70% (37). The inhibition pattern of DON was very similar to that of cycloheximide: both inhibitors affected the recovery of cells from trypsin treatment but did not completely inhibit recovery. Also, neither inhibitor had an effect on collection of recovered cells. Dimethyl sulfoxide (DMSO) alone, the solvent for DON, had no effect on the collection of cells, and the inhibitions produced by DON and cycloheximide were dose-dependent processes. Two metabolic inhibitors, DNP and KCN, had a strong inhibiting effect on recovery and on collection of recovered cells (Tables I and II). These results were the same as the observations of Um-

TABLE I
Effect of Inhibitors on Freshly Trypsinized Cells

Inhibitor	Control rate		Exp. rate		Exp. rate / Control rate
	% cells collected/h/flask	*SD*	*% cells collected/h/flask*	*SD*	*%*
Cycloheximide (5 μg/ml)	8.6	1.0	4.7	0.9	54
DON (15 μg/ml)	15.4	2.1	9.9	1.0	64.2
DNP (2.5 mM)	13.8	1.4	1.9	0.4	13.7
KCN (16 μM)	12.2	1.8	2.3	0.7	18.8
4°C	9.85	2.2	0.2	0.3	2

Collection of freshly trypsinized cells was not linear. Therefore, "rates" were determined from the 0-h to 4-h time-point and averaged as percentage cells collected/h/flask. Each rate represents the mean of six flasks with the SD given. A percentage comparison between control and experimental rates is given by calculating the mean experimental rate/mean control rate for each inhibitor.

TABLE II
Effect of Inhibitors on Recovered Cells

Inhibitors	Control rate		Correlation coefficient	Exp. rate		Correlation coefficient	Exp. rate / Control rate
	%/h	*SD*		*%/h*	*SD*		*%*
Cycloheximide (25 μg/ml)	30.2	1.8	0.96	28.9	1.1	0.93	96
DON (15 μg/ml)	14.5	3.2	0.86	17.5	2.2	0.97	120
DNP (2.5 mM)	37.5	3.8	0.96	3.3	2.4	0.52	8.8
KCN (16 μM)	21.8	1.0	0.90	4.0	0.75	0.49	18.3
Ouabain (50 μM)	24.3	5.6	0.90	24.1	4.8	0.93	99
4°C	16.6	0.9	0.98	0.01	0.47	0.11	0.06

Rates of collection were determined by linear regression analysis of at least 12 flasks processed per experimental or control, using four time-points per experiment. Correlation coefficients reflect the relative linearity of rates of collection over a 2-h collection experiment. Control rates vary between experiments for two reasons: either the number of collecting aggregates was different, or the rate of speed of rotation was different. Within an experiment, these parameters were the identical for an experimental and control set of flasks. Approx. percentage control was determined by dividing the mean experimental rate by mean control rate.

breit and Roseman (40) (though their culture conditions and assay methods were somewhat different). We also found that inhibition in the presence of DNP or KCN was more pronounced when glucose was not included in the culture medium during collection. Low temperature completely inhibited both recovery and collection of recovered cells. Ouabain, at concentrations known to inhibit Na^+K^+ ATPase (41), had no effect on collection of recovered cells.

Effects of Colchicine and Cytochalasin B on Collection of Recovered Cells

We found colchicine and cytochalasin B to affect what we observe as recovery (both inhibit collection by better than 70% when added to freshly trypsinized cells). Collection of recovered cells is not affected by 10^{-4} M colchicine (Table III).

Cytochalasin B, by contrast, inhibits collection of recovered cells, and the inhibition is reversible (Table III). One criticism of the use of cytochalasin B is that, in addition to its effect on actinlike microfilaments, cytochalasin B also inhibits sugar transport and therefore may affect glycoprotein metabolism as well (8, 33). Our data obtained with DON show that even when glycoprotein metabolism is affected, recovered cells still collect at a rate that is the same as that for the control, untreated cells. Therefore, the effect of cytochalasin B suggests that actinlike microfilaments could be important for some aspect of the collection of cells to aggregates.

Antigenic Differences between Freshly Trypsinized and Recovered Cells

The data presented above suggested that materials important for adhesion were replaced at the cell surface during recovery. To approach the identification of these materials, we asked whether we could distinguish differences between freshly trypsinized cell surfaces and recovered cell surfaces. We isolated membranes of recovered cells by the methods of Merrell and Glaser (23). Antiserum was raised in rabbits to the crude recovered cell membrane preparations. The antiserum was tested to determine whether there were immunologically recognizable differences between freshly trypsinized cells and recovered cells.

First, the antiserum was tested by indirect immunofluorescence by the methods of Goldschneider and Moscona (9). The antiserum was absorbed twice at a 1:1 cell to serum volume, and then tested by serial dilution in microtiter plates. Cells were added to the wells, incubated at 4°C for 20 min, and then washed twice by precipitation and resuspension in PBS. The cells were then incubated in preabsorbed goat antirabbit immunoglobulin labeled with fluorescein. As shown in Table IV, absorption of the antiserum with freshly trypsinized cells had little effect on the immunofluorescence titer when the serum was tested on recovered cells. Recovered cells, by contrast, readily absorbed away most of the specific immunofluorescence activity of the antiserum. Recovered cerebrum cells absorbed some but not all of the retina cell-surface binding activity. These results therefore suggested that the antiserum detected antigenic differences between the cell-surface of freshly trypsinized cells and that of recovered cells.

We next attempted to determine whether any of the recovered cell antigens might be involved in adhesion. We tested the absorbed antiserum for its ability to affect collection of cells to aggregates

TABLE III

Effect of Colchicine and Cytochalasin B on Rate of Collection

Inhibitor	Control rate		Correlation coefficient	Exp. rate		Correlation coefficient	Exp. rate / Control rate
	%/h	*SD*		*% cells collected/flask*	*SD*		*%*
Colchicine (1×10^{-4} M)	23.5	1.4	0.96	21.3	1.8	0.90	92
Cytochalasin B (5 μg/ml)	28.5	3.1	0.94	10.8	2.7	0.79	37
After removal of cytochalasin B (5 μg/ml)	28.5	3.1	0.94	26.3	1.8	0.97	92

Inhibitors were added to recovered cells 1 h before addition of the cells to collecting aggregates. Inhibitors were also present during collection. Cytochalasin B was removed after a 1-h incubation. Rates are expressed in percentage cells collected/flask/h for 2 h. Correlation coefficients reflect the degree of linearity of a collection. Other calculations are as described in Table II.

TABLE IV

Immunofluorescence of Neural Retina Membrane Antiserum

Serum	Absorbed with	Cell type tested	Immunofluorescence reciprocal titer
Anti-NR^{10}*	Unabsorbed	NR^{10} (recovered)	3,200
Anti-NR^{10}	NR^{10} (trypsinized)	NR^{10} (trypsinized)	24–48
Anti-NR^{10}	NR^{10} (trypsinized)	NR^{10} (recovered)	1,600
Anti-NR^{10}	NR^{10} (recovered)	NR^{10} (recovered)	100
Anti-NR^{10}	Cerebrum cells	NR^{10} (recovered)	400–800
Anti-NR^{10} Fab fragments	NR^{10} (trypsinized)	NR^{10} (recovered)	1,600

* NR^{10} = 10-day embryonic chick neutral retina cells.

(Fig. 2; Table V). We assumed that if antibodies were to bind to adhesive sites and affect collection, there should be a dose dependency in the response. Accordingly, recovered cells were incubated in several dilutions of antiserum (preabsorbed with freshly trypsinized cells). The cells were then resuspended in EI-DNase and added to collecting aggregates. Fig. 2 shows a dose-dependent inhibition of collection suggesting that at least some of the antigens identified by the antiserum might be important in adhesion. Neither the preimmune serum nor antiserum that had been absorbed with recovered cells had the inhibitory effect (Table V). We have also tested Fab fractions on recovered cell collection (Table V); again, there is a dose-dependent inhibition but only during the first half-hour after incubation with antibody fragments.

In a separate study on sea urchin cells (19), whole antiserum to membranes enhanced collection of cells to aggregates. We have also recently characterized a new antiserum to neural retina membranes, and it too enhances collection of recovered cells rather than inhibiting their collection. We do not know, at present, why one antiserum enhances collection specifically while another inhibits collection specifically. One serum may be agglutinating the cells to cause apparent enhancement, though many other possibilities exist.

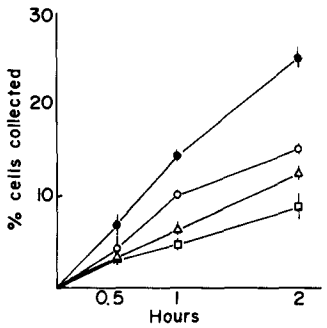

FIGURE 2 Effect of retina membrane antiserum on the collection of cells to retina cell aggregates. Tissues were dissociated with trypsin and cells were recovered for 4 h. The cells were incubated for 20 min at 10°C in three dilutions of antiserum that had been absorbed 2 × against freshly trypsinized cells. After being incubated in serum, the cells were resuspended in EI and DNase, and added to flasks containing collecting aggregates. Each point represents the mean of three replicate determinations (1.8×10^5 ^{3}H-labeled cells/flasks; 0.3 dpm/cell). ● = control; ○ = 1/6 dilution; △ = 1/3 dilution; □ = 1/1 dilution.

Specificity of Collection of Freshly Trypsinized vs. Recovered Neural Retina Cells

In the chick, the retinal projection of axons to the surface of the optic tectum shows a high degree of specificity (3, 15, 16). Several in vitro studies have shown that retina cells from the dorsal or ventral neural retina adhere preferentially to the ventral or dorsal optic tectum, respectively (3, 4, 15, 16, 30). A double gradient of specificity molecules has been proposed to account for the specificity observed: a dorsal-ventral gradient that has a low sensitivity to trypsin, and a ventral-dorsal gradient that has a higher trypsin sensitivity (3, 15, 30).

We have examined the retino-optic tectum system using our aggregate collection assay, and our results confirm the observations cited above (Fig. 3). We compared the rate of collection of either dorsal neural retina cells or ventral neural retina cells to the surface of 24-h aggregates of dorsal or ventral optic tectum. Freshly trypsinized cells of the dorsal retina adhered preferentially to aggregates of the ventral optic tectum (Fig. 3*a*). By contrast, freshly trypsinized cells of the ventral neural retina did not show any adhesion preference during the first few hours of collection to

TABLE V

Effect of Antiserum on Collection

Serum	Concn	Absorbed by	Control rate		Correlation coefficient	Exp. rate		Correlation coefficient	Exp. rate / Control rate
			%/h	SD		% cells collected/flask/h	SD		%
Anti-R^{10}	1:1	Trypsinized R^{10}	30.5	3.4	0.94	17.5	0.8	0.98	57
Anti-R^{10}	1:3	Trypsinized R^{10}	30.5	3.4	0.94	22.5	1.1	0.98	73
Anti-R^{10}	1:6	Trypsinized R^{10}	30.5	3.4	0.94	25.4	2.3	0.96	83
Anti-R^{10}	1:1	Recovered R^{10}	22.9	1.6	0.97	21.0	1.6	0.97	92
Preimmune	1:1	Trypsinized R^{10}	30.1	2.5	0.97	27.9	1.1	0.99	93
*Fab fragments	–	–	25.4	1.7	0.98	12.8	1.3	0.98	50

Recovered cells were incubated with serum at the given dilutions for 20 min at 10°C. The cells were then diluted with EI + DNase and added in 0.2-ml aliquots to collecting flasks. Serum was preabsorbed either with trypsinized NR^{10} cells or with recovered cells.

*Fab fragments of the anti-R^{10} serum were added to cells at a protein concn of 2 mg/ml. The 50% inhibition of adhesion was present only for the first 30 min of collection. After that, the inhibitory effect disappeared to background by 2 h.

aggregates of optic tectum (Fig. 3*c*). If the retina cells were recovered for 5 h before addition to the collecting aggregates, both the dorsal retina and the ventral retina showed a preference for aggregates of the opposite tectal half. In other words, dorsal retina was less sensitive to the effects of trypsin than ventral retina; dorsal retina cells showed a preference for ventral optic tectum aggregates long before the ventral cells showed any preference for dorsal optic tectum.

Initially, we were surprised that the aggregate collection procedure showed retino-tectal specificity at all. Previous studies had shown the specificity in intact optic tecta (3, 4, 15, 16, 30). Our results therefore suggest two possibilities: either (*a*) the dorsal-ventral recognition pattern is present on all cells of the embryonic optic tectum, or (*b*) in the 24-h period of aggregation there is a rearrangement of tectal cells sufficient to confer a dorsal or ventral specificity to the surface of the aggregates.

DISCUSSION

After trypsinization of embryonic neural retina cells, several hours of recovery are necessary to restore the full capacity for adhesion as measured by the collection assay. During this time, antigens are inserted at the cell surface, and these antigens apparently are necessary for the high rate and specificity of adhesion. When the antigens are blocked by specific antibodies, collection is inhibited. Replacement of the adhesion-related antigens is affected by cycloheximide and by DON, an inhibitor of amino sugar metabolism. Low temperature and inhibitors of ATP also inhibit the recovery process. After recovery has occurred, adhesion can take place in the absence of protein synthesis and in the absence of DON-inhibitable sugar metabolism. The adhesion measured is a temperature-dependent and energy-dependent process.

The finding that cell-surface repair is necessary for adhesion of trypsinized cells is perhaps not surprising. Many cell-surface phenomena, e.g., specific receptors, have been shown to require recovery time after trypsinization for restoration of function (6, 7, 32, 34, 36). We have taken advantage of the recovery process in order to distinguish between those processes involved in the synthesis of cell-surface molecules and those processes involved in the formation of cell-cell interactions. Which adhesion-related materials are replaced during the recovery process? Populations of antigens identified in this study satisfy many requirements that suggest involvement in adhesion: (*a*) the antigens are present on the cell surface; (*b*) they are removed or greatly reduced in number by trypsinization; (*c*) they reappear during the period of time when biological activity is restored; and (*d*) specifically absorbed antiserum binds to these antigens and prevents adhesions from being formed.

The antigens identified may relate to one or more of the "factors" described by several workers using neural retina cells (1, 12, 14, 21, 32). In those studies, culture supernates were shown to contain materials which were synthesized by neural retina cells and apparently turned over by

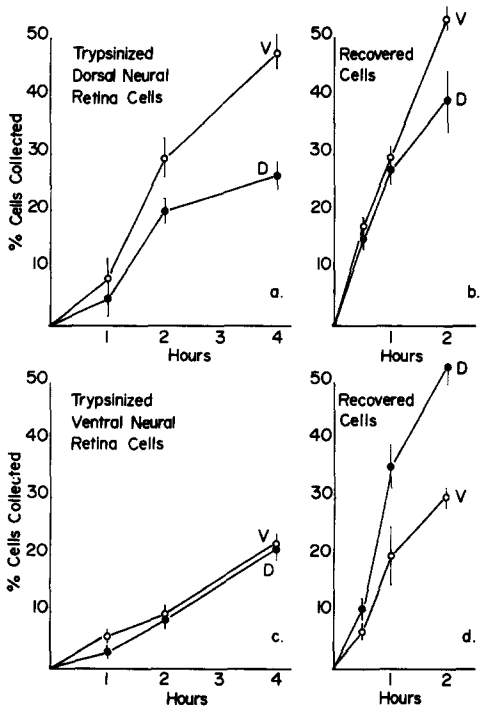

FIGURE 3 Collection of dorsal neural retina cells (*a* and *b*) or ventral neural retina cells (*c* and *d*) to the surface of aggregates of dorsal optic tectum or ventral optic tectum. In Fig. 3*a* and *c*, freshly trypsinized cells were added to flasks containing 24-h aggregates. In Fig. 3*b* and *d*, cells that had been incubated in EI-DNase plus 2% fetal calf serum for 5 h were added to flasks containing 24-h aggregates. All tissues were dissected from 10-day embryos. The cells were labeled with [^{3}H]leucine as given in Materials and Methods. Collecting aggregates were labeled with [^{14}C]leucine. Since the collecting aggregates in these experiments were of two types, collection rates were normalized by comparing total ^{14}C per flask. This normalization assumes that the rate of incorporation of [^{14}C]leucine is the same for the dorsal optic tectum as it is for the ventral optic tectum. Each graph represents the result of a single experiment in which three replicates per time point were used. Fig. 3*a*, *b*, and *c* show results that were similar in four of four trials on each combination. Fig. 3*d* shows results that were similar in two of four trials. The other two trials with cells of recovered ventral neural retina showed a preference for dorsal optic tectum, but the rates of collection were not significantly different from the rate of collection to ventral optic tectum.

the cells. These factors have been shown to bind specifically to the surface of retina cells, and apparently they promote or enhance adhesion of freshly trypsinized cells (1, 12). The variety of factors described is puzzling, however, and it is not clear at the molecular level how the factors affect adhesion, although several intriguing hypotheses have been advanced (2, 10, 25, 29, 32). These hypotheses frequently suggest involvement of cell-surface adhesive sites, so it is quite possible that at least some of the antigens identified in the present study are the putative adhesive sites.

Other studies have shown that retina-specific antisera affect cell interactions (9). As yet, however, little is known about the function of the cell-surface antigens identified in this study or in other studies, so it might be instructive to consider what the results shown in Fig. 2 and in Tables IV and V actually tell us. The specificity of the inhibition and the controls suggest that at least some of the antigens identified in this study might be involved in the neural retina adhesion process. It is possible, however, that we are identifying antigens that are themselves not involved in adhesion but are closely bound to adhesion molecules such that there is a steric effect. Such a relationship is difficult to disprove. Another problem to be resolved has to do with the variety of antigens involved in the inhibition. Here the possibilities are numerous. One possibility is that a single neural retina antigen suffices for the adhesion interactions of all retina cells. This, however, would not be the case if the data on retino-tectal specificity are considered. It is also possible that a number of unrelated cell-surface molecules contain similar side chains that are used for purposes of adhesion. Rutishauser et al. (32) have suggested that cell-surface-adhesion molecules become activated for adhesion by proteolytic removal of a portion of the molecule. Other experiments have implicated the action of glycosyl transferases in altering cell-surface-adhesion-related molecules (28, 35). These examples of possible secondary alterations of cell-surface materials further complicate the evidence and point out that the current level of understanding of cell-adhesion-related molecules is fragmentary. The availability of an approach for isolation of antigens as suggested by this study may help to resolve the confusion that presently exists.

When it has been possible to measure the presence of receptors on the cell surface, it has been observed that a 4- to 6-h recovery period is required for the receptors to be replaced after enzymatic digestion (34, 41). In the absence of known measurable receptors for cell adhesion, evidence for post-trypsin repair has been indirect. In a number of assays, a lag period has been observed before cells become adhesive (26, 36, 38, 42).

The length of the lag period observed is rather short (up to 1 h) when compared to the 4- to 6-h replacement time observed for measurable surface receptors after trypsinization. Recently, however, several reports have indicated that longer periods of time are necessary for complete repair of cell surfaces in adhesion assays. Cassiman and Bernfield (6) report that up to 24 h are required for tissue culture cells to recover fully from trypsinization. McGuire (22) reports that 6–8 h of recovery are required after papain treatment and that several hours are required for recovery after very mild trypsin treatment of embryonic liver cells. If a long period of repair is necessary for adhesion in these examples, why is it that only a short lag is observed in many assays and that no lag is observed before the initiation of adhesion in other assays (13, 26, 38, 42)? It is quite likely that the differences depend upon the technique. For example, we have compared our aggregate collection technique with the monolayer assay of Walther et al. (42). Using the Walther assay, we confirmed their observation that recovery does not appear to be necessary (Fig. 4, *inset*). Using the same freshly trypsinized cell suspension in the aggregate collection assay, we observed the sigmoid curve characteristic of recovering cells (Fig. 4, *inset*). Our interpretation of the difference in technique is given in Fig. 4. This interpretation assumes a threshold effect for adhesion interactions. In some assay systems that have a low threshold for successful interactions, a majority of freshly trypsinized cells could adhere with minimal recovery due to incomplete removal of adhesive sites by trypsin. The aggregate collection system uses a rotary shaker method and rather stringent washing procedures to remove "reversible" (40) or "provisional" (17) contacts, and it measures, therefore, relatively stable interactions between cells and collecting aggregates. Apparently, many adhesive sites are removed by trypsin, and the aggregate collection assay is sensitive to that reduction.

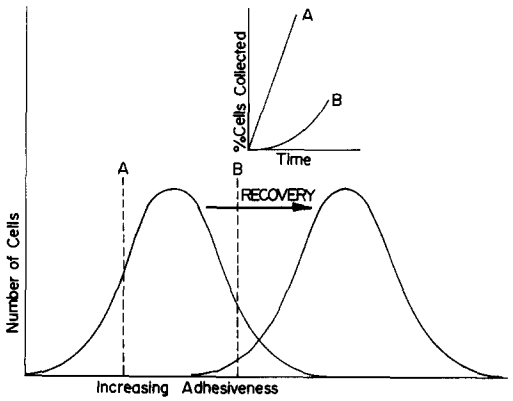

FIGURE 4 Model explaining the recovery data. The bell-shaped curve assumes that freshly trypsinized cells will be distributed over a range of "adhesiveness" which is defined as the relative ability to interact with another cell or group of cells. *A* and *B* represent two "thresholds." For any assay, conditions can be adjusted so that more or fewer cells can form adhesions. Cells falling below a threshold in an assay will not form successful cell interactions. If an assay were to have threshold *A*, then the observed rate of adhesion of freshly trypsinized cells might look like *A* in the small graph. If an assay were to have threshold *B*, then the curve *B* would be expected for freshly trypsinized cells adhering with time and recovery. If the population of cells were to recover before being tested, then one would expect both assays *A* and *B* to show linear rates of adhesion.

Our observations on retino-tectal specificity were unexpected, but they too point out the need for cell-surface recovery after trypsinization. One might also take advantage of the apparent difference in trypsin sensitivity between the dorsal and the ventral neural retina to explore the double gradient theory proposed by Barbera (3), Barbera et al. (4), Marchase (15), Marchase et al. (16), and Roth and Marchase (30).

The demonstration of cell-surface repair as a prerequisite for specific cell interactions, and an immunological probe to monitor the repair, offers an approach for the isolation and identification of cell-surface-adhesion-related molecules. This approach is one of several that promise an explanation of the molecular nature of at least some aspects of the adhesion process. Such an explanation is necessary before many of the hypotheses concerning morphogenesis and other cellular processes can be confirmed and extended.

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